

The Differences in the Involvements of Loci of Promoter Region and Ile50Val in Interleukin-4 Receptor α Chain Gene between Atopic Dermatitis and Japanese Cedar Pollinosis

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ABSTRACT

Background: Atopic dermatitis (AD) and Japanese cedar pollinosis (JCP) are common chronically allergic diseases associated with the activation of T-helper 2 cells. Recent studies have shown that polymorphisms in the genes for IL-4 receptor α chain (IL4RA) may contribute to susceptibility of AD and JCP, although the differences in the involvements of loci of IL4RA gene between AD and JCP are unclear. In this study, we investigated the role of polymorphisms in IL-4RA gene in conferring susceptibility to the development of AD and/or JCP using a family analysis and an association analysis in a Japanese population.

Methods: Five polymorphisms in the IL-4RA gene, C-3223T, T-1914C, T-890C, Ile50Val and Glu375Ala, have been genotyped using PCR-based methods in 75 trios families, including 15 AD families, 30 JCP families, and 30 families with combination of AD and JCP in the family analysis. Forty-five AD, 60 JCP and 125 control children constituted the association study.

Results: The transmission disequilibrium test showed that the allele of Ile50 was significantly transmitted to children with JCP alone ($p < 0.05$). Haplotype analysis showed that the -3223T/Ile50 haplotype was preferentially transmitted to both AD ($p < 0.01$) and JCP children ($p < 0.01$), while that the C-3223/Ile50 haplotype was preferentially transmitted to only JCP children ($p < 0.01$). The association study showed that -3223T and haplotype of -3223T/Ile50 were associated with AD children, but not with JCP. Ile50 was associated with both AD and JCP.

Conclusions: Our data suggest that -3223T and the -3223T/Ile50 haplotype were risk factors for AD. Ile50 allele seems to be involved in both JCP and AD. Interactions of the IL-4RA loci may play a role both conferring susceptibility and modulating severity of AD.

KEY WORDS

atopic dermatitis, haplotype, IL-4, Japanese cedar pollinosis, polymorphism

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INTRODUCTION

Atopic dermatitis (AD) is a common, chronic and recurrent dermal inflammation with erythema, papule and scale usually on the whole body that occurs with increasing prevalence. It is characterized by hyperactivated cytokines of helper T cell subset 2 and high level of serum IgE.^{1,2} AD often accompanies other atopic diseases such as asthma, Japanese cedar pollinosis (JCP) and conjunctivitis³⁻⁵ and is known to develop by the interaction of genes and environment.⁶⁻⁹

JCP, defined as a type I allergic disease as well as AD, has recently remarkably increased. It is one of the most common forms of hay fever in Japan that develops ocular and nasal symptoms paroxysmally upon contact with Japanese cedar pollen in spring.^{10,11} The seasonal symptoms are conjunctival itching and allergic rhinitis such as sneezing, excessive nasal secretion and nasal congestion. JCP is also considered to be caused by the interaction of genes and environment.¹²

Interleukin 4 (IL4) is a pleiotropic cytokine produced by mast cells, basophils and T cells and plays a central role in IgE-dependent inflammatory reactions.¹³ IL4 is central to B cells' switching to IgE antibody production, and to the maturation of T-helper (Th) cells to the Th2 phenotype. IL4 operates through the IL4 receptor (IL4R), a heterodimeric complex comprising the IL4R α chain (IL4RA) and γ c chain. Ober and his colleagues¹⁴ conducted a systematic search for variations in the IL4RA gene and found 13 polymorphic variants in the coding region, including 7 variants that resulted in amino acid substitution. Izuhara *et al.*¹⁵ have demonstrated that the prevalence of Ile50 is higher than that of 50Val in individuals with atopic asthma, especially in those in childhood in the Japanese. The Ile50Val variant of IL4RA upregulates IgE synthesis and is associated with atopic asthma.¹⁶

In our previous study, the Ile50Val variant of IL4RA has been demonstrated to be associated with the development of JCP.¹² Hesselmar *et al.*¹⁷ have compared the relationships of C-3223T variant in IL4RA between AD and pollen allergy, since AD accompanies JCP.⁴ However, the contribution of IL-4RA polymorphisms to AD, JCP or both remain to be elucidated well. Given the central role of the IL-4RA pathway in AD and JCP, we have investigated the role of five common polymorphisms in the IL-4RA gene, three loci in promoter region (T-890C, T-1914C, C-3223T), two in coding region (Ile50Val, Glu375Ala), in conferring susceptibility to the development of AD and/or JCP using a family analysis and an association analysis in a Japanese population.

METHODS

SUBJECTS

We recruited 950 subjects in Makioka-cho, a country

side area of Yamanashi Pref., and Shinagawa area of Metropolitan Tokyo, Japan, who voluntarily received the examination for the diagnosis of the AD and JCP and for the study of its etiology. All subjects received a self-administered questionnaire regarding their history of allergic diseases, i.e., JCP, asthma, and AD as well as nasal and conjunctival symptoms: sneezing, nasal discharge, itching of nasal mucosa or conjunctiva, watering eyes. They underwent intradermal test (IDT) and nasal provocation test (NPT) using house dust, mite, ragweed, and Japanese cedar (*Cryptomeria japonica*). The diagnosis of AD was based on standard criteria.^{18,19} The diagnosis of JCP was based on the questionnaire, IDT, NPT, and a history of cedar pollinosis. The criteria for diagnosis of cedar pollinosis were as follows: 1) under drug therapy or immunotherapy for cedar pollinosis, 2) at least one positive condition of IDT or NPT in subjects with symptoms. We performed a family study and an association one using the subjects who were recruited in Makioka-cho and Shinagawa area. All subjects were fully informed of the protocol and had given their informed consent before the experiment. This study was approved by the Ethics Committee on Experimentation of Kanazawa University.

Family Study

Sixty children under 16 years old, diagnosed with cedar pollinosis according to the criteria and their 80 parents including 48 cedar pollinosis patients and 32 healthy subjects constituted the subjects of this study. As 40 of 60 affected children were siblings, our subjects included 40 families. The average ages \pm standard deviation of 60 children and their 80 patients were 10.4 ± 3.20 years and 38.8 ± 3.42 years, respectively. Total 15 children included fifteen children who were diagnosed as AD, 30 children as JCP, and 30 children as the combination of AD and JCP. The average age \pm standard deviation for 75 children and their 150 patients were 10.8 ± 3.10 years and 39.5 ± 33.4 years, respectively. There was no statistically significant difference in the age among the three children groups of AD, JCP, and the combination. The average IgE value \pm standard error of AD, JCP and controls were 133 ± 25.8 , 278 ± 42.1 , and 37.5 ± 4.45 (U/ml), respectively. The IgE values in AD and JCP (both $p < 0.001$) were significantly higher than that in controls.

Association Study

We performed an association study in which 45 children with AD alone and 60 children with JCP alone were selected from unrelated subjects in Makioka-cho and Shinagawa area. One-hundred twenty five children were set as the control corresponding to the patients by using balance match method for age and sex. The controls did not show either symptoms of AD, pollinosis, condition of IDT or NPT. All subjects of the association study were selected differently

Table 1 Transmission disequilibrium test (TDT) for IL4RA gene polymorphisms in 75 families including children with AD, JCP and combination of AD and JCP

Allele	AD (15 families)		JCP (30 families)		Combination (30 families)	
	Transmitted	Not	Transmitted	Not	Transmitted	Not
-3223T	9	3	11	6	12	4
-1914C	7	2	11	5	11	3
-890C	1	0	0	1	1	0
Ile50	9	3	15*	3*	8	2
Glu375	4	1	6	2	6	2

* $p = 0.0095$ and 0.048 without and with correction for multiple comparisons by Bonferroni adjustment, respectively.

from those of the family study. The average ages \pm standard deviation of AD, JCP patients and control groups in the association study were 10.2 ± 1.80 , 10.5 ± 1.92 and 10.1 ± 2.54 years, respectively. The average IgE value \pm standard error of AD, JCP and controls were 142 ± 27.6 , 261 ± 30.0 , and 43.4 ± 4.90 (U/ml), respectively. The values for AD and JCP (both $p < 0.001$) were significantly higher than 43.4 ± 4.90 in controls.

Total serum IgE was measured by enzyme immunoassay (TOSOH, Tokyo, Japan).

GENOTYPE

DNA was extracted from peripheral blood leukocytes using the Automatic DNA Isolation System (KURABO, Tokyo, Japan). Amplification was performed using a Takara LA Taq (TAKARA, Kyoto, Japan). The final reaction mixture (25 μ l) consisted of 50 ng of genomic DNA (1 μ l), 2.5 μ l Taq buffer, 2 μ l dNTP, 0.125 μ l Taq, 2 μ l MgCl₂ and 1 μ l IL4RA variant specific primer pairs. The specific primer pairs used were as follows: T-890C of promoter region of IL4RA, forward 5'-TGTGTTCTGAATCCCAGCTCC-3' and reverse 5'-CCTGGCAACTACCCTATAAG-3'; T-1914C of promoter region of IL4RA, forward 5'-GACTTATCTTTACTGTCACT-3' and reverse 5'-TTAGTAGACATGAGGTTTCA-3'; C-3223T of promoter region of IL4RA, forward 5'-CGAAAGGCTTGGAAGAAGT-3' and reverse 5'-TAGACCCACCTCATAGGGCTA-3'; exon 5 of IL4RA, forward 5'-CGGAATTCCGAGGCCCACACGTGT-3' and reverse 5'-CGCTGGGCTTGAAGGAG-3'; exon 12 of IL4RA, forward 5'-ATCAGCGTGGTGCGATGTGT-3' and reverse 5'-GAATGAGGTCTTGGAAGG-3'. For IL4RA polymorphisms, the PCR protocol consisted of a pre-PCR heat activation step (95°C, 5 min) followed by 25 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min), and final cycle extension at 72°C, 7 min. PCR products were visualized after agarose gel electrophoresis and ethidium bromide staining. PCR products were purified using of Microcon (Millipore, Bedford, MA, USA).

We analyzed five polymorphisms of IL4RA gene which Hackstein²⁰ cited three loci in the promoter re-

gion (T-890C, T-1914C, C-3223T), the two in coding region (Ile50Val, Glu375Ala). Nucleotide variants in the promoter region are numbered according to the relative position to exon 1 and the numbers of amino acids correspond to the IL4RA mature protein which is a membrane-bound receptor. Ile50Val (extracellular variant) and Glu375Ala (intracellular variant) are in exon 5 and exon 12, respectively.

DNA SEQUENCING

Direct DNA sequencing of PCR products was performed on an ABI Prism 377 Genetic Analyzer (Perkin Elmer) using the ABI Prism Big Dye Terminator DNA sequencing kit (Perkin Elmer).

STATISTICS

TDT was analyzed using Genehunter (version 2.1). The calculations of haplotype, linkage disequilibrium and Hardy-Weinberg equilibrium were computed by employing the Arlequin software package (version 2.0), which implemented the EM algorithm to estimate maximum likelihood haplotype frequencies. The contributions of the IL4RA loci to the phenotypes performed by the association study were assessed by χ^2 test and the odds ratio, as was analyzed by SPSS (version 17.0J). P values obtained by the TDT and the association study were corrected for multiple comparisons (Bonferroni adjustment according to the number of comparisons made: $P_{cor} = P\text{-value} \times 5$ (number of analyzed IL4RA variants)). The comparisons of total IgE value among AD, JCP and controls, and among polymorphisms in the IL4RA genes were analyzed using one-way analysis of variance, followed by the multiple comparisons by Dunnett test. P values less than 0.05 were regarded as statistically significant.

RESULTS

TDT

Table 1 showed the results of TDT in five polymorphisms in children with AD or JCP and combination of AD and JCP. The allele of Ile50 was significantly transmitted to children with JCP alone even after correction for multiple comparisons by Bonferroni ad-

Table 2 Linkage disequilibrium parameter (D') among C-3223T, T-1914C and Ile50Val in children with 45 AD and 60 JCP

Allele	AD (45 children)		JCP (60 children)	
	T-1914C	Ile50Val	T-1914C	Ile50Val
C-3223T	0.286*	0.528***	0.178	0.563***
T-1914C	-	0.197	-	0.230*

* $p < 0.05$, *** $p < 0.001$.**Table 3** Frequencies of haplotypes of C-3223T, T-1914C and Ile50Val in children with 45 AD and 60 JCP

Haplotype			AD	JCP
A	B	C		
0	0	0	0.300	0.352
1	0	1	0.175	0.155
1	1	1	0.172	0.120
0	0	1	0.097	0.060
0	1	0	0.091	0.074
1	1	0	0.069	0.067
0	1	1	0.056	0.173
1	0	0	0.039	0

A-0, C-3223; A-1, -3223T; B-0, T-1914; B-1, -1914C; C-0, 50Val; C-1, Ile50.

justment ($p < 0.05$). Nine children with AD whose Ile 50 allele were transmitted showed a higher proportion as compared to 3 AD children whose Ile50 were not, as was not significant. Similarly, 8 children with the combination of AD and JCP whose Ile50 was transmitted showed proportion as compared to 2 children without transmission, as was not significant.

LINKAGE DISEQUILIBRIUM ANALYSIS

Significant linkage disequilibrium was observed between C-3223T and T-1914C ($p < 0.05$), and between C-3223T and Ile50Val ($p < 0.001$) in AD children. In JCP children, significant linkage disequilibrium was observed between C-3223T and Ile50Val ($p < 0.001$), and between T-1914C and Ile50Val ($p < 0.05$) (Table 2). Allele frequencies of C-3223T, T-1914C and Ile50 Val did not deviate from expected Hardy-Weinberg equilibrium examined by χ^2 test ($p = 0.66, 0.47$, and 0.40 in C-3223T, T-1914C, and Ile50Val for AD; $p = 0.21, 0.15$, and 0.71 in C-3223T, T-1914C, and Ile50Val for JCP, respectively).

HAPLOTYPE ANALYSIS AND HAPLOTYPE TDT

As we found significant linkage disequilibrium in three loci (C-3223T, T-1914C, and Ile50Val), we conducted haplotype analysis of them. The frequencies of three-locus haplotypes were shown in Table 3. The frequencies of haplotypes other than these loci were very low. In AD and JCP, the most common haplo-

Table 4 Haplotype TDT in 45 families of children with AD and 60 with JCP

Haplotype	Observed/Expected	
	AD	JCP
-3223T/Ile50	16/10**	17/9**
-3223T/50Val	3/6	4/7
C-3223/Ile50	11/8	14/8**
C-3223/50Val	4/6	4/6

** $p < 0.01$.

type was allele 0-0-0 which means C-3223, T-1914 and Val50. We performed haplotype TDT based on the frequencies (Table 4). The -3223T/Ile50 haplotype was preferentially transmitted to both AD and JCP children ($p < 0.01$), while the C-3223/Ile50 was preferentially transmitted to only JCP children ($p < 0.01$).

ASSOCIATION STUDY

We recognized significant differences in allele frequencies of two loci in IL4RA gene between controls and AD or JCP (Table 5). The allele frequency in -3223T, 44.4% in patients with AD showing a odds ratio (95% confidence interval) of 1.98 (1.62-2.42) was significantly higher than that in controls, even after correcting for multiple comparisons by Bonferroni adjustment ($p < 0.05$). However, we did not find a significant difference in the C-3223T between controls and JCP. On the contrary, the allele frequencies in Ile50 in both AD and JCP were significantly higher than that in control, even after adjustment by Bonferroni method, showing odds ratios of 0.44 (0.36-0.55) ($p < 0.05$) and 0.44 (0.37-0.52) ($p < 0.01$), respectively. The frequency of -3223T/Ile50 haplotype in AD, but not in JCP, was significantly higher than in control ($\chi^2 = 31.6, p < 0.001$).

There were significant differences in IgE value of two loci in IL4RA gene among polymorphisms. The multiple comparison by Dunnett method demonstrated that the IgE value in C/T allele of C-3223T were significantly higher than that in C/C allele ($p < 0.01$). The IgE value in Ile/Ile allele were significantly higher than that in Val/Val ($p < 0.01$).

DISCUSSION

Many researchers have tried to identify variants of the IL-4RA genes and have examined their associations with asthma, atopic dermatitis and cedar pollinosis.²¹ Systematic research has demonstrated polymorphic variants in the coding regions in the IL-4RA gene, including 7 variants resulting in amino acid substitution. Ile 50 Val,²² Ser 487 Pro,²³ and Gln551Arg²¹ have been reported to be involved in the risk for hyper-IgE syndrome, atopic dermatitis, and the asthma phenotype. Our previous study also demonstrated that the polymorphism of Ile50Val in the IL-

Table 5 Association study for L4RA gene polymorphisms in 45 children with AD, and 60 children with JCP compared with 125 controls

Gene	Allele	Control		AD		JCP		IgE value (U/ml) [‡]
		Number	Frequency [†]	Number	Frequency [†]	Number	Frequency [†]	
C-3223T	C/C	73	28.8	17	44.4	29	28.3	89.9 ± 10.5
	C/T	32		16		28		162.5 ± 26.1**
	T/T	20		12		3		125.9 ± 33.4
Odds ratio (5%-95% CI)				1.98 (1.62-2.42)		0.98 (0-100000)		
p, corrected p value				0.010, 0.0498		0.976, 1		
T-1914C	T/T	70	26.4	23	30	29	28.33	120 ± 16.4
	T/C	44		17		28		123.6 ± 19.4
	C/C	11		5		3		95.3 ± 15.6
Odds ratio (5%-95% CI)				1.19 (0.33-4.36)		1.10 (0.076-16.0)		
p, corrected p value				0.604, 1		0.7895, 1		
T-890C	T/T	120	2	41	4.44	58	1.67	121.4 ± 12.0
	T/C	5		4		2		79.3 ± 28.1
	C/C	0		0		0		119.4 ± 11.5
Odds ratio (5%-95% CI)				2.28 (0.25-20.7)		0.83 (0-100000)		
p, corrected p value				0.392, 1		0.852, 1		
Ile50Val	Ile/Ile	52	37.6	31	21.1	38	20.8	149.7 ± 17.4##
	Ile/Val	52		9		19		104 ± 18.8
	Val/Val	21		5		3		35 ± 7.8
Odds ratio (5%-95% CI)				0.44 (0.36-0.55)		0.44 (0.37-0.52)		
p, corrected p value				0.0066, 0.0329		0.0018, 0.0092		
Glu375Ala	Glu/Glu	110	6	42	3.33	55	4.17	119.8 ± 12.5
	Glu/Ala	15		3		5		115.1 ± 28.3
	Ala/Ala	0		0		0		119.4 ± 11.5
Odds ratio (5%-95% CI)				0.54 (0.04-6.61)		0.68 (0.03-16.8)		
p, corrected p value				0.4875, 1		0.628, 1		

[†] Frequency of minor allele (%), [‡] Mean ± standard error, Statistical difference by Dunnett test as compared to the value of C-3223T,

***p* < 0.01 and the value of 50Val, ##*p* < 0.01.

4RA gene was responsible for the development of JCP.¹² The substitution of Ile for Val augmented STAT6 activation, proliferation, and transcription activity of the promoter by IL-4,²⁴ resulting in enhancement of IgE synthesis and development of JCP. In our association study, we also recognized that the polymorphism of Ile50Val in the IL-4RA gene was associated with JCP and AD. Our results were supported by the many other previous results that IL-4RA Ile50 was closely associated with JCP.¹² Tanaka *et al.*^{25,26} suggested that AD patients with high IgE level was associated with IL-4RA Ile50. Therefore, our results examining AD patients showing a high IgE level is considered to agree well with the assumption that IL-4RA Ile50 plays a role in the production of IgE in AD patients.

Ober *et al.*¹⁴ suggested that variations outside the coding region of the IL-4RA gene influence susceptibility to atopy and asthma. The polymorphisms in the 5' promoter region of the IL-4RA were reported,²⁰ e.g. IL-4RA C-3223T and IL-4RA T-1914C are the distal

promoter polymorphisms. Usually the promoter polymorphisms might influence the transcription level of the gene. Indeed, the promoter polymorphism, C to T at position -3223 in the IL-4R gene was shown to influence the levels of the soluble IL-4RA (sIL-4RA) in serum in Germany.²⁰ Secreted forms of sIL-4RA occur naturally in both mice and humans and have been described to act as antagonists to IL-4.²⁷ In vivo treatment with sIL-4R, in a mouse model, prevented the development of hypersensitivity and airway hyper-responsiveness.²⁸ While Hytonen *et al.*²⁹ revealed the C-3223T allele associated with lower serum levels of sIL-4R and patients carrying the T allele also had severe atopic asthma in Swedish Caucasians. In the present study, we found novel strong linkage disequilibria between C-3223T and Ile50Val in AD. Hosomi and his colleagues³⁰ demonstrated that the IL-4RA gene polymorphisms in the distal promoter region, C-3223T were significantly associated with AD in Japanese population. Novak *et al.*³¹ showed the prevalence of the -3223T in IL4RA gene tended to be

higher in AD than in nonatopic donors. Our findings showed a higher IgE value in the subjects with C-3223T as compared to C-3223C allele, suggesting that -3223T as well as Ile50 are associated with a higher IgE. Taken together, these findings suggest that the C-3223T promoter polymorphism in the IL-4RA gene plays a key role in development of AD.

In addition, we found that the -3223T/Ile50 haplotype was preferentially transmitted to AD children, while that the C-3223 and -3223T/Ile50 haplotypes were preferentially transmitted to JCP children. Furthermore, the association study showed that -3223T and haplotype of -3223T/Ile50 were associated with AD children, but not with JCP. On the contrary, Ile50 was associated with both AD and JCP. These results suggest that presence of 3223T allele and the -3223T/Ile50 haplotype were risk factors for AD, and that Ile 50 allele was for both JCP and AD. It is well known that AD accompanies JC and that AD patients complicated with JCP clinically tend to have more severe AD symptoms.⁴ An exacerbation of AD lesions during the Japanese cedar pollen season has been reported.³² From these findings, it seems that combination of AD with JCP aggregates symptoms in AD patients. Both patients³³ and dogs³⁴ with AD showed high concentration of allergen-specific IgE for Japanese cedar pollen. We know that dogs sensitized by Japanese cedar pollen allergens become to have AD.³⁵ Taken together, JCP are thought to be exacerbation factors for AD. Our results showing the involvement of -3223T/Ile50 haplotype in AD suggest that the concomitant of the two polymorphisms, -3223T and Ile50 develop not only JCP and also AD.

In response to the problem of "spurious associations" to describe an association between a phenotype and a marker locus when the marker is unlinked to any causative loci, Spielman *et al.*³⁶ proposed the transmission disequilibrium test (TDT), which uses the genotypes of parents of affected individuals. In the present study we used TDT, which method was different from that of the association study between the promoter region of IL-4RA and AD by Hosomi *et al.*³⁰ The TDT is not susceptible to spurious associations, but our samples were not so many trios families. More subjects should be examined in further studies using our procedures.

In conclusion, our results demonstrated the involvements of -3223T allele and the -3223T/Ile50 haplotype in AD and that of Ile50 in AD and JCP, suggesting that children with both -3223T and Ile50 may develop AD. It seems likely that children with Ile50 alone develop JCP. Interactions of the IL-4RA loci may play a role both conferring susceptibility and modulating severity of AD.

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